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# Adhesive Properties of Osteopontin: Regulation by a Naturally Occurring Thrombin-Cleavage in Close Proximity to the GRGDS Cell-binding Domain

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Osteopontin (OPN) is a secreted adhesive glycoprotein with a functional glycine-arginine-glycine-aspartate-serine (GRGDS) cell-binding domain. An interesting feature of OPN structure is the presence of a thrombin-cleavage site in close proximity to the GRGDS region. Cleavage of OPN by thrombin is likely to be of physiological importance, because cleavage of blood plasma OPN occurs naturally after activation of the blood coagulation pathway. To investigate functional consequences of OPN cleavage by thrombin, cell attachment and spreading assays were performed with uncleaved and cleaved forms of OPN. For all cell lines examined, thrombin-cleaved OPN promoted markedly greater cell attachment and spreading than uncleaved OPN. Cell attachment and spreading on thrombin-cleaved OPN was inhibited both by the soluble GRGDS peptides and an OPN-specific antibody raised to the GRGDS domain of OPN, thus implicating the GRGDS region in mediating the increased cell attachment and spreading observed on thrombin-cleaved OPN. Because the GRGDS sequence in OPN is only six residues from the thrombin-cleavage site, the data suggest the possibility that thrombin cleavage allows greater accessibility of the GRGDS domain to cell surface receptors. To investigate receptors that recognize uncleaved and thrombin-cleaved OPN, affinity chromatography was performed on placental extracts; the cell surface integrin $\alpha_v\beta_3$ bound to columns constructed either with native or thrombin-cleaved OPN and was selectively eluted from each with soluble GRGDS peptide and EDTA. Moreover, adhesion assays performed in the presence of $\alpha_v\beta_3$ blocking monoclonal antibody LM609 identified $\alpha_v\beta_3$ as a major functional receptor for thrombin-cleaved OPN. Several lines of evidence suggest that cleavage of OPN by thrombin occurs in vivo, such as in tumors and at sites of tissue injury, and adhesion assay data presented here indicate that such cleavage is important in the regulation of OPN function.

## INTRODUCTION

Osteopontin (OPN)[1] is a secreted adhesive glycoprotein with a functional glycine-arginine-glycine-aspartate-serine (GRGDS) cell-binding domain (Oldberg *et al.*, 1986). When immobilized to substrata, OPN promotes attachment and spreading of a variety of cell types (Oldberg *et al.*, 1986; Somerman *et al.*, 1989; Brown *et al.*, 1992; Chambers *et al.*, 1993) through interactions with the $\alpha_v\beta_3$ cell surface integrin (Ross *et al.*, 1993) and possibly other integrins as well. OPN has an unusual tissue distribution for a GRGDS-containing protein; it is expressed prominently in bone (Yoon *et al.*, 1987; Nomura *et al.*, 1988; Chen *et al.*, 1993) but also at the apical surface of many epithelial cells that line the gastrointestinal, genitourinary, and respiratory tracts, as well as epithelial cells of salivary ducts and glands, sweat ducts and glands, and lactating breast (Brown *et al.*, 1992). Osteopontin also circulates in blood

[1] Abbreviations used: BSA, bovine serum albumin; OPN, osteopontin; SDS, sodium dodecyl sulfate.

plasma (Senger *et al.*, 1988) and is expressed by at least some ganglion cells (Mark *et al.*, 1988; Swanson *et al.*, 1989; Brown *et al.*, 1992). Additionally, OPN has been reported to be expressed by activated T-cells (Singh *et al.*, 1990b) and macrophages (Senger *et al.*, 1988; Miyazaki *et al.*, 1990). Collectively, the presence of OPN in bone, on epithelial surfaces and in their secretions, in blood, and in ganglion cells suggests multiple functions for this protein (reviewed by Denhardt and Guo, 1993). Moreover, expression of OPN by cells of the immune response after activation, and by wounded arterial smooth muscle cells in response to injury (Giachelli *et al.*, 1993), suggest that OPN may function importantly in inflammation and tissue repair.

An interesting feature of OPN structure, which we described previously, is the presence of a thrombin-cleavage site within six amino acids of the GRGDS cell-binding domain (Senger *et al.*, 1989a,b). Purified OPN is cleaved at this site by catalytic, naturally occurring, concentrations of purified thrombin; moreover, OPN present in whole blood plasma is identically cleaved in the course of blood coagulation (Senger *et al.*, 1988). Thus, enzymatic cleavage of OPN, in close proximity to the GRGDS cell-binding domain, occurs under physiological conditions and could serve importantly to regulate the function of this protein. Furthermore, OPN and thrombin are likely to be present together wherever the blood coagulation pathway is activated, such as in wounds, tumors, and at sites of inflammation (reviewed by Dvorak, 1986). Therefore, to determine the functional consequences of OPN cleavage by thrombin, we have studied the adhesion of a variety of cell types to native and thrombin-cleaved OPN and investigated interactions between both forms of this protein and the cell surface integrin $\alpha_v\beta_3$.

## MATERIALS AND METHODS

### *Purification of OPN and Thrombin Cleavage*

Rat OPN was purified from the serum-free culture medium of a rat tumor cell line (ts B77-Rat 1) as described previously (Senger *et al.*, 1989b). Human OPN was purified from breast milk also as described previously (Senger *et al.*, 1989b), except that for high-pressure liquid chromatography we employed a Beckman (Fullerton, CA) Spherogel TSK phenyl-5PW column (7.5 mm × 7.5 cm). Before loading, protein was diluted with 8 vol of 2 M ammonium sulfate. All OPN bound to the column under these conditions and was eluted subsequently with a 60 ml linear gradient of 2 M ammonium sulfate to 0.01 M sodium phosphate pH 6.8. Purified OPN typically eluted at ~0.7 M ammonium sulfate.

Cleavage of purified OPN in solution by purified thrombin was performed as described (Senger *et al.*, 1989b) and verified by polyacrylamide gel electrophoresis (PAGE) (see Figure 2). Thrombin cleaves OPN at a single site as determined by N-terminal amino acid sequence analyses (Senger *et al.*, 1989b). To remove thrombin after OPN cleavage, we employed an affinity matrix constructed by adsorbing 10 U antithrombin III (Sigma Chemical, St. Louis, MO) to 2 ml heparin-Sepharose (Pharmacia, Piscataway, NJ), followed by washing with phosphate-buffered saline. One hundred microliters of this matrix was sufficient for removing 1 U thrombin; thrombin removal was verified with the Kabi S2238 chromagenic substrate assay (Kabi Pharmacia, Franklin, Ohio). Before use in adhesion assays, OPN and thrombin-cleaved OPN were filter sterilized by passing through Millex GV filters (Millipore, Bedford, MA) without loss of protein.

### *Cell Lines, Cell Culture, and Cell Attachment and Spreading Assays*

HT1080 (human fibrosarcoma), T24 (human bladder carcinoma), HISM (human intestinal smooth muscle), and HFL-1 (human fetal lung fibroblast) cell lines used in this study were obtained from the American Type Culture Collection (Rockville, MD). They were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. For cell attachment and spreading assays, HT1080 cells were harvested with 1 mM EDTA in Hanks' balanced salt solution without divalent cations. However, EDTA alone was insufficient for harvesting of HFL-1, T24, and HISM cells that adhere strongly under normal culture conditions. Therefore, they were subjected to brief trypsinization (0.05% trypsin, 0.5 mM EDTA); cells obtained adhered to fibronectin and vitronectin within 60 min indicating that they were suitable for adhesion assays. For these assays, as described below, cells were resuspended at a density of $1–3 \times 10^5$ ml in serum-free DMEM containing 5 mM glutamine and 10 mg/ml bovine serum albumin (BSA) (Sigma Chemical).

Cell attachment and spreading assays were performed according to the following protocols. Although plastic is readily coated with rat OPN, we found that passive adsorption of human milk OPN to plastic or glass was not generally reliable, possibly because human milk OPN carries a higher negative charge than rat OPN at neutral pH. Therefore, we coupled human OPN covalently to glass or plastic substrata according to a previously described method devised by others for adhesive proteins (Werb *et al.*, 1989). Glass coverslips (12 mm) were used primarily for cell spreading assays; these were coated with aminopropyltriethoxysilane (Sigma), rinsed, activated with 0.25% glutaraldehyde, and washed extensively with sterile saline. Coverslips were placed in 24-well tissue culture plates and incubated overnight at 37°C with 1 ml of OPN or thrombin-cleaved OPN (each at 6 μg/ml) or human plasma fibronectin or vitronectin (each at 10 μg/ml) (Telios Pharmaceuticals, San Diego, CA). After washing, remaining protein binding sites were blocked (2 h, 37°C) with 10 mg/ml BSA (heat denatured at 70°C for 1 h). In some experiments, we compared binding of human OPN and thrombin-cleaved human OPN (radiolabeled with [$^{125}$I]) to the activated glass coverslips and found them to be very similar although thrombin-cleaved OPN bound with slightly lower efficiency (~15% less). Nonetheless, coverslips coated with thrombin-cleaved OPN promoted substantially more extensive cell attachment and spreading than those coated with uncleaved OPN, despite the slightly reduced binding efficiency of thrombin-cleaved OPN (see RESULTS). We also employed an alternative protocol in which we cleaved OPN in situ after it was covalently bound; OPN was first coupled to the activated glass coverslips, remaining protein binding sites were blocked with BSA, and then coverslips were incubated with either saline alone or saline plus thrombin (2 U/ml, 45 min, 37°C).

For quantitative cell attachment assays with human OPN, we primarily employed Costar 96-well strip plates (#2388, Cambridge, MA) with an amine-derivatized surface for covalent binding of protein. Wells were activated with glutaraldehyde and incubated with OPN followed by BSA, as for glass coverslips. Thrombin-cleavage of covalently bound OPN also was as described above. For quantitative cell attachment assays with rat OPN, Costar high protein binding polystyrene 96-well strip plates were coated with rat OPN at a concentration of 10 μg/ml (noncovalent, passive adsorption), followed by BSA, and, where indicated, followed by incubation with thrombin as described above. Before plating, cells were labeled overnight with 25 μCi/ml L-[$^{35}$S]-methionine in standard culture medium, harvested, and suspended in serum-free medium also as described above. At the end of the assay, unattached cells were removed by gentle washing with DMEM.

### *Peptides, Antibodies, and Immunoprecipitation*

GRGDSP, GRGDS, and control GRADSP and GRGESP peptides were purchased from Telios Pharmaceuticals. GRGDSPK peptide (with lysine residue added for coupling to Sepharose), the human OPN peptide VPTVDTYDGRGDSVVYGLR(C) (with cysteine added for conjugation, see below), and the human $\beta_3$ integrin C-terminal peptide (C)ANNPLYKEATSTFTNITYRGT, designed according to strategy employed by Marcantonio and Hynes (1988), were synthesized by Multiple Peptide Systems (San Diego, CA).

The OPN peptide containing the GRGDS sequence and the $\beta_3$ peptide representing C-terminal sequence were conjugated through cysteine residues to keyhole limpet hemocyanin, rabbits were immunized, and antisera were derived as described (Sioussat *et al.*, 1993). To obtain affinity-purified antibody, peptide was covalently coupled to cyanogen bromide-activated Sepharose (Pharmacia, Piscataway, NJ) and antibody bound and eluted as described (Brown *et al.*, 1992). In enzyme-linked immunoadsorbent assays (ELISAs) the OPN peptide antibody demonstrated specific binding to the OPN peptide and human OPN protein, but no detectable binding to human collagen type I, human fibronectin, or human vitronectin (all from Telios). The $\beta_3$ peptide antibody was specific for $\beta_3$ as demonstrated by staining of a single protein species on immunoblots with expected mobility (Mr 105 000 with reducing conditions, Mr 90 000 nonreduced).

$\alpha_v\beta_3$ monoclonal antibody LM609 ascites (Cheresh, 1987) were kindly provided by Dr. David Cheresh, Scripps Clinic and Research Foundation, La Jolla, CA, and antibody was purified with the AffiGel protein A MAPS II Kit (Bio-Rad Laboratories, Richmond, CA). Control mouse IgG was purchased from Sigma and repurified with the same procedure. The $\alpha_v\beta_3$ antibody LM609 was employed in adhesion assays only. For staining of $\alpha_v$ on immunoblots (see below), we used a monoclonal antibody to human $\alpha_v$ (clone VNR139, from Telios) at 1:1000 dilution followed by incubation with rabbit antiserum to mouse IgG (Sigma) at 1:200 dilution for subsequent detection with [$^{125}$I]-protein A (see below). The $\beta_3$ peptide rabbit antiserum was used in immunoblotting at 1:200 dilution.

Immunoprecipitation of $\beta_3$ integrins from affinity column fractions (see below) was performed with the $\beta_3$ peptide antiserum and protein A Sepharose beads (Pharmacia). Beads were washed and resuspended in 4 vol of buffer (0.01 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid pH 8.0, 0.15 M NaCl, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 0.1% NP40). Two hundred microliters of column fraction was incubated for 1 h with 10 $\mu$l antiserum, followed by 1 h with 100 $\mu$l protein A bead slurry; beads with bound antibody and antigen then were washed four times with buffer (above).

### *PAGE and Immunoblotting*

For protein electrophoresis in the absence of sodium dodecyl sulfate (SDS), we employed 4–20% (wt/vol) gradient polyacrylamide gels. Gels, sample buffer, and electrode buffer all contained 0.025 M tris (hydroxymethyl) aminomethane (Tris), 0.192 M glycine pH 8.3. PAGE in the presence of SDS (for immunoblotting) was performed as described previously (Laemmli, 1970). Immunoblotting also was performed as described (Towbin *et al.*, 1979). After incubation with appropriate antibodies (see above), blots were incubated with ~0.2 $\mu$Ci/ml [$^{125}$I]-Protein A (5 $\mu$Ci/$\mu$g) (Dupont New England Nuclear, Wilmington, DE), washed, and subjected to autoradiography. Typical exposure times required for visualization of integrin subunits was 8–18 h.

### *Affinity Chromatography of Human Placental Integrins*

Affinity supports were constructed by covalent coupling of human OPN, thrombin-cleaved human OPN, and synthetic peptides (see legend to Figure 4) through primary amine groups to cross-linked agarose beads (either cyanogen bromide–activated Sepharose [Pharmacia] or AminoLink coupling gel [Pierce Chemical, Rockford, IL]). Coupling was carried out according to manufacturers' recommendations; efficiency of coupling was generally 50–60% for OPN and thrombin-cleaved OPN and 80% for the synthetic peptides.

Placental extracts were prepared essentially as described (Pytela *et al.*, 1987; Gailit and Ruoslahti, 1988). Eighty grams of human placenta were extracted at 4°C for 30 min in lysis buffer consisting of Tris-buffered saline (TBS) (50 mM Tris HCl pH 7.3, 150 mM NaCl) together with 1 mM $MgCl_2$, 1 mM $MnCl_2$, 100 mM octyl-$\beta$-D thioglucopyranoside (OTG), and 2 mM protease inhibitor AEBSF (4-(2-aminoethyl)-benzenesulfonylfluoride from Calbiochem, La Jolla, CA). The extract was centrifuged at 10 000 × *g* for 15 min at 4°C, and 20 ml aliquots of supernatant were incubated batchwise for 16 h at 4°C with each of the four affinity supports (~1.5 ml packed volumes, see Figure 4 legend) that had been equilibrated in wash buffer (TBS containing 25 mM OTG, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 2 mM AEBSF). After incubation with placental extract, each affinity support was washed three to four times batchwise with wash buffer, poured into columns, and washed again with ~8 vol of wash buffer. Bound integrins were eluted with GRGDS peptide (1.0 mg/ml) in wash buffer and/or with 20 mM disodium EDTA in wash buffer without divalent cations (Gailit and Ruoslahti, 1988).

## RESULTS

### *Cell Adhesion to Native and Thrombin-cleaved OPN*

To compare cell adhesion to native and thrombin-cleaved OPN, we performed cell attachment and spreading assays with both human and rat OPN and a variety of human cell lines including HFL-1 (fibroblasts), T24 (bladder carcinoma), HT1080 (fibrosarcoma), and HISM (intestinal smooth muscle). For comparisons of native OPN with thrombin-cleaved OPN, assays were performed according to two protocols: first, by immobilizing equal quantities of native or cleaved OPN on substrata, followed by incubation with BSA to block remaining protein binding sites; and second, by coating substrata with OPN, incubating with BSA, and subsequently cleaving substratum-bound OPN in situ with added thrombin (see MATERIALS AND METHODS). Adhesion assays performed according to both protocols gave indistinguishable results; typical experiments are presented in Figure 1 and Table 1. With all of the cell lines and with both adhesion assay protocols, and regardless of whether OPN was covalently bound to substratum or passively adsorbed (Table 1), thrombin-cleaved OPN promoted more extensive cell attachment and spreading than uncleaved OPN. Moreover, HT1080 cells, which did not attach detectably to native OPN in these assays, consistently demonstrated attachment to thrombin-cleaved OPN. In general, we have found that substrata coated with OPN do not promote the rapid (<60 min) cell attachment and spreading observed on substrata coated with fibronectin. Nonetheless, for all cell types studied here, some attachment to thrombin-cleaved OPN was evident within 4 h, and T24 and HFL-1 cells attached within 90 min and 60 min, respectively (Table 1). Thus, differences between cell adhesion to thrombin-cleaved and native OPN were readily apparent within 60 min of plating of HFL-1 cells, within 90 min for T24 cells, 2 h for HISM cells, and 4 h for HT1080 cells. To eliminate possible complications because of

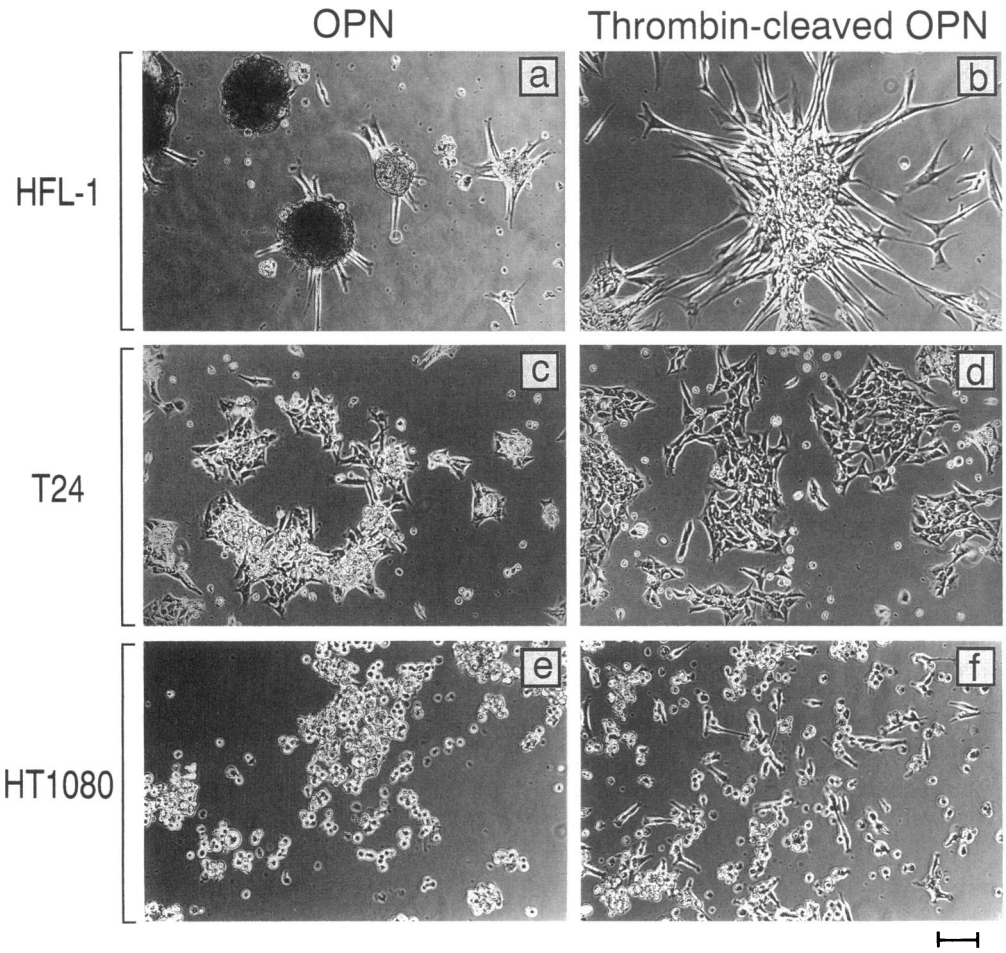


**Figure 1.** Attachment and spreading of HFL-1, T24, and HT1080 cells on uncleaved OPN and thrombin-cleaved OPN. For experiments with HFL-1 cells shown here, glass coverslips were coupled with human OPN (6 μg/ml) followed by BSA (10 mg/ml), and then incubated with saline alone (a) or saline with thrombin (2 U/ml) (b) followed by extensive washing; cells were then added. For experiments with T24 and HT1080 cells, glass coverslips were coupled with human OPN (6 μg/ml) followed by BSA (c and e) or with thrombin-cleaved OPN (6 μg/ml) from which thrombin had been removed (d and f) (see MATERIALS AND METHODS). Increased cell attachment and spreading on thrombin-cleaved OPN in comparison with uncleaved OPN was observed consistently in experiments performed according to both protocols. We observed no cell attachment to substrata coated with BSA alone (not shown). HFL-1 cells were photographed at 23 h, T24 cells at 18 h, and HT1080 cells at 5 h after plating. Bar, 24 μm.

cell-secreted proteins, in several experiments we preincubated and assayed cells in the presence of cycloheximide, at concentrations of the drug sufficient to abolish protein synthesis, and determined that such cells also attach more rapidly to thrombin-cleaved OPN in comparison with native OPN (Table 1, experiment B).

Findings presented in Figure 1 and Table 1 cannot be explained by the presence of contaminating thrombin or, alternatively, by more efficient coating of substrata by thrombin-cleaved OPN in comparison with coating of substrata by native OPN. For assays employing OPN that was thrombin-cleaved before coating of substrata (Figure 1, Table 1), all detectable thrombin was removed by repurification of OPN (see MATERIALS AND METHODS). Furthermore, we investigated the possibility that thrombin might promote cell attachment and spreading and found for the cell types employed here and at thrombin concentrations used, that thrombin markedly inhibited cell attachment to cleaved OPN such that attachment was less than that observed on native OPN alone. Moreover, we compared coating of substrata with radioiodinated OPN and thrombin-cleaved OPN and found that native and cleaved OPN coated substrata with very similar efficiencies (see MATERIALS AND METHODS). Finally, we consistently observed much more extensive cell attachment and spreading on substrata that was coated first with native OPN, next incubated with BSA to block residual protein binding sites, and subsequently incubated with thrombin to cleave substratum-bound OPN (Figure 1, Table 1). Therefore, we conclude that markedly increased cell adhesion to thrombin-cleaved OPN in comparison with native OPN, as demonstrated with these assays, is a direct consequence of enzymatic cleavage of OPN by thrombin.

### *Structure of Thrombin-Cleaved OPN: Evidence for Dissociation of the Two Derived Polypeptides*

As noted above, we have determined previously with amino acid sequence analyses that OPN contains a single thrombin-cleavage site and that this site is only six amino acid residues from the carboxy-terminal side of the GRGDS cell-binding domain. The two thrombin-generated fragments appear to be of very similar size because they are not easily resolved from each other in SDS-containing polyacrylamide gels; both fragments derived from human OPN have electrophoretic mobil-

**Table 1.** Cell attachment to OPN in comparison with attachment to thrombin-cleaved OPN

| Experiment | Cell line | # of assays | Time | % cell binding to: BSA alone | OPN | Thrombin-cleaved OPN |
|---|---|---|---|---|---|---|
| A. Human OPN covalently coupled to substrata | HT 1080 | (3) | 5 h | 4.0 ± 1.7 | 3.7 ± 2.1 | 20.3 ± 8.5 |
| | HISM | (4) | 4 h | 2.3 ± 0.5 | 39.8 ± 10.1 | 80.8 ± 11.0 |
| | T24 | (5) | 18 h | 4.0 ± 1.2 | 16.8 ± 3.7 | 54.4 ± 4.6 |
| | HFL-1 | (6) | 1.5 h | 3.5 ± 0.6 | 34.3 ± 8.0 | 75.2 ± 5.9 |
| B. Human OPN covalently coupled to substrata, cells treated with cycloheximide | T24 | (3) | 1.5 h | 2.4 ± 0.6 | 27.0 ± 3.5 | 94.1 ± 6.3 |
| | HFL-1 | (3) | 1.5 h | 6.7 ± 2.5 | 22.3 ± 1.0 | 77.7 ± 3.5 |
| C. Rat OPN passively adsorbed to substrata | T24 | (6) | 1.5 h | 2.7 ± 0.3 | 16.0 ± 7.6 | 79.3 ± 15.5 |
| | HFL-1 | (5) | 1.0 h | 1.8 ± 0.2 | 36.9 ± 3.2 | 70.8 ± 7.7 |

Attachment assays were performed with $^{35}S$-labeled cells on substrata prepared as indicated below; detailed descriptions are provided in MATERIALS AND METHODS. The data are expressed as percentage cell binding ± SD; percentage cell binding was calculated by dividing d.p.m. bound (to a well with the specified substratum) by average d.p.m. bound to control wells coated with fibronectin and multiplying the quotient by 100. Experiments (A) with HT1080 cells were performed with glass coverslips to which equal quantities of human OPN or thrombin-cleaved human OPN were coupled covalently. Experiments (A and B) with HISM, T24, and HFL-1 cells were performed with plastic to which human OPN had been coupled covalently; where indicated, OPN was subsequently cleaved in situ by thrombin. Also, experiments (B) were performed with cells pretreated with the protein synthesis inhibitor cycloheximide (20 μg/ml) for 1 h and maintained in the inhibitor during the assay (1.5 h). Experiments (C) were performed with plastic which was coated with rat OPN (noncovalent adsorption); where indicated rat OPN was subsequently cleaved in situ by thrombin.

ities in SDS corresponding to a molecular weight ($M_r$) of ~35 000 (Senger *et al.*, 1989b). Moreover, the two fragments likely share other physiochemical properties, because we have not succeeded thus far in separating them by ion exchange or reverse phase high resolution chromatography. OPN contains no cysteine residues that might contribute to intrachain bonding, and consequently, the OPN fragments migrate similarly in SDS polyacrylamide gels, whether or not reducing agents are present. However, to investigate the possibility that the two fragments remain associated through noncovalent interactions, we subjected thrombin-cleaved OPN to electrophoresis in the absence of SDS and other denaturing agents. As shown in Figure 2, thrombin-cleaved OPN migrated in nondenaturing polyacrylamide gels as a single band of unresolved polypeptides corresponding to ~$M_r$ 30 000. If the two OPN fragments had remained associated, mobility of thrombin-cleaved OPN, under these electrophoretic conditions, would be similar to that of uncleaved OPN that corresponded to ~$M_r$ 67 000 in this system (Figure 2). Thus electrophoretic analysis of thrombin-cleaved OPN under nondenaturing conditions indicated that the two polypeptides generated by thrombin-cleavage did not remain associated.

### *Function of the GRGDS Cell-binding Domain After Thrombin-Cleavage*

Cell attachment to native OPN has been shown previously to be inhibited by soluble GRGDSP peptides but not by control GRGESP peptides (Oldberg *et al.*, 1986), thus providing evidence for the importance of its GRGDS domain in promoting cell attachment. Similarly, and as expected if the GRGDS domain remained functional in thrombin-cleaved OPN, we observed complete inhibition of HT1080 cell attachment to thrombin-cleaved OPN in the presence of soluble GRGDSP peptide (300 mM) but little or no inhibition in the presence of an equal concentration of control GRADSP or GRGESP peptide.

To investigate further the importance of the GRGDS domain for cell binding to thrombin-cleaved OPN, we raised a rabbit antibody to a 20 amino acid synthetic peptide representing the GRGDS domain of human OPN. As determined by ELISAs, antibody to this peptide was specific for OPN and did not bind detectably to other GRGDS-containing proteins (see MATERIALS AND METHODS). When employed in adhesion assays, this antibody markedly inhibited cell attachment and spreading on thrombin-cleaved OPN (Figure 3), providing direct evidence for the importance of the GRGDS region in mediating cell attachment to thrombin-cleaved OPN. Similarly, this antibody also substantially blocked cell attachment to native OPN.

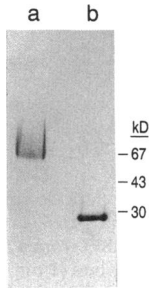


**Figure 2.** Electrophoresis of OPN and thrombin-cleaved OPN in nondenaturing conditions. Proteins were subjected to electrophoresis on a 4–20% gradient polyacrylamide gel in Tris-glycine buffer pH 8.3 toward the anode. (a) Four micrograms native human OPN, (b) 4 μg thrombin-cleaved human OPN; molecular weight markers: BSA (67 000), chicken ovalbumin (43 000), and bovine erythrocyte carbonic anhydrase (30 000). Gel was stained with Coomassie Brilliant Blue R-250.

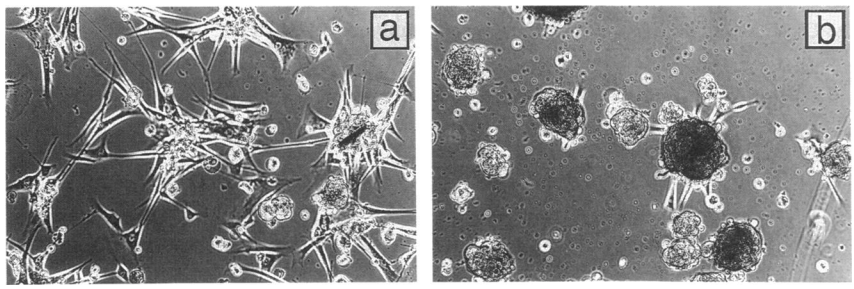


**Figure 3.** Inhibition of HFL-1 cell attachment to thrombin-cleaved OPN by antibody raised to a 20 amino acid peptide containing the GRGDS cell-binding domain of OPN. HFL-1 cells were plated on thrombin-cleaved OPN as in Figure 1 in the presence of either (a) 30 μg/ml control rabbit IgG or (b) 30 μg/ml affinity-purified rabbit antibody to a 20 amino acid synthetic peptide corresponding to the GRGDS domain of OPN. This peptide antibody substantially inhibits cell attachment and spreading on thrombin-cleaved OPN (b). Cells were photographed 18 h after plating. Bar, 24 μm.

### *Involvement of the $\alpha_v\beta_3$ Cell Surface Integrin in Binding Native OPN and Thrombin-cleaved OPN*

Others have demonstrated previously that the LM609 blocking monoclonal antibody to integrin $\alpha_v\beta_3$ inhibits interactions between osteoclasts and OPN, thus implicating $\alpha_v\beta_3$ as an important osteoclast receptor for OPN (Ross *et al.*, 1993). We sought to determine both if $\alpha_v\beta_3$ binding to OPN could be demonstrated with affinity chromatography and if the thrombin-cleaved form of OPN also bound $\alpha_v\beta_3$. Affinity supports were constructed through covalent coupling of either human OPN or thrombin-cleaved human OPN to beads of cross-linked agarose. We found that cells attached to such beads but not to control beads, indicating that OPN still promoted cell adhesion after covalent coupling to the agarose support. Next, detergent extracts of human placenta were incubated with the OPN-agarose beads, followed by extensive washing of the beads, and elution of the bound proteins with buffer containing 20 mM EDTA (see MATERIALS AND METHODS). Eluted proteins were analyzed for $\alpha_v$ and $\beta_3$ integrin subunits by electrophoresis and immunoblotting; a typical experiment is shown in Figure 4. As determined with antibody staining, both $\alpha_v$ and $\beta_3$ subunits bound and were eluted from columns covalently coupled to GRGDSPK peptide (RGD in Figure 4), the 20 amino acid OPN peptide corresponding to the GRGDS domain of OPN (OPN-RGD), OPN, and thrombin-cleaved OPN (OPN cl.). Moreover, the only major protein bands seen on silver-stained polyacrylamide gels were coincident with the $\alpha_v$ and $\beta_3$ bands identified on immunoblots. To demonstrate direct association between $\alpha_v$ and $\beta_3$ subunits, which were bound and eluted from OPN and thrombin-cleaved OPN, we performed immunoprecipitations with $\beta_3$ antibody and subjected the immunoprecipitates to immunoblotting with $\alpha_v$ and $\beta_3$ antibodies. As shown in Figure 5, $\beta_3$ antibody precipitates (lanes i) of eluted proteins contained both $\alpha_v$ and $\beta_3$ subunits (as detected by immunoblotting) indicating that $\alpha_v\beta_3$ heterodimers bound to OPN and thrombin-cleaved

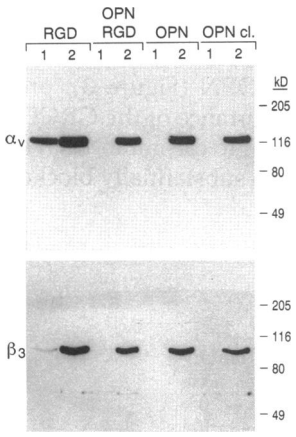


**Figure 4.** Immunoblots of placental integrins eluted from affinity columns. Columns (1.5 ml) were constructed with covalent coupling of the following peptides or proteins to cross-linked agarose beads: RGD, control RGDSP(K) peptide (10 mg); OPN RGD, 20 amino acid OPN peptide containing GRGDS (20 mg); OPN, uncleaved OPN (10 mg); and OPN cl., thrombin-cleaved OPN (10 mg). Lanes 1 and 2 represent 80 μl volumes of successive 1.0-ml fractions collected from the indicated columns after extensive washing and immediately after addition of elution buffer containing 20 mM EDTA (see MATERIALS AND METHODS). Electrophoresis was carried out under reducing conditions on 7.5% polyacrylamide gels containing SDS, electroblotted, and stained with antibody to $\alpha_v$ or $\beta_3$, as indicated. $\alpha_v$ and $\beta_3$ were also present in fractions eluted from all of the columns with buffer containing soluble GRGDS peptide (1 mg/ml), although in lower amounts than in fractions eluted with 20 mM EDTA.

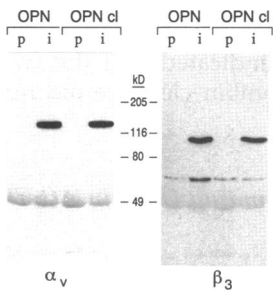


**Figure 5.** Identification of $\alpha_v\beta_3$ heterodimers in fractions eluted from affinity columns constructed with OPN and thrombin-cleaved OPN. Fractions eluted from columns as in Figure 4 were subjected to immunoprecipitation with preimmune serum (lanes p) or $\beta_3$ immune serum (lanes i). Subsequently, immunoprecipitates were subjected to electrophoresis (reducing conditions) followed by electroblotting and staining with either antibody to $\alpha_v$ or $\beta_3$ as indicated. Both $\alpha_v$ (125 kDa) and $\beta_3$ (105 kDa) bands are present specifically in $\beta_3$ immune precipitates (lanes i) but not control precipitates with preimmune serum (lanes p) demonstrating that $\alpha_v\beta_3$ heterodimers were eluted from both OPN and thrombin-cleaved OPN (OPN cl.) affinity columns.

OPN affinity columns. Neither of the subunits were present in control immunoprecipitates (Figure 5, lanes p).

To investigate the importance of $\alpha_v\beta_3$ for cell attachment and spreading on thrombin-cleaved OPN, we performed adhesion assays with several cell lines in the presence of $\alpha_v\beta_3$ blocking monoclonal antibody LM609 (kindly provided by Dr. David Cheresh). As shown in Figure 6, this $\alpha_v\beta_3$ antibody substantially inhibited HFL-1 cell attachment and spreading on thrombin-cleaved OPN and also inhibited the minimal HFL-1 cell attachment and spreading that occurred on uncleaved OPN. We observed similar inhibition of T24 cell attachment and spreading on thrombin-cleaved and uncleaved OPN with this antibody; there was no inhibition by control antibody in any of the experiments. In addition, we performed quantitative cell attachment assays with HT1080 cells in the presence and absence of $\alpha_v\beta_3$ antibody LM609. In four experiments, similar to those we performed with HT1080 cells but without antibody (see Table 1), we observed an average of 85.4% ± 2.4% inhibition of HT1080 cell attachment to thrombin-cleaved OPN in the presence of 5 μg/ml of LM609 antibody but no inhibition in the presence of control antibody. Thus, our data indicate that, for all three cell lines examined (i.e., HFL-1, T24, HT1080), $\alpha_v\beta_3$ is a major functional receptor for thrombin-cleaved OPN.

## DISCUSSION

In this study, we have demonstrated with adhesion assays that thrombin-cleaved OPN, in comparison with uncleaved OPN, promotes markedly increased cell attachment and spreading regardless of whether the proteins are covalently bound or passively adsorbed to substrata. Several human cell lines were tested including a carcinoma (T24), fibrosarcoma (HT1080), fibroblasts (HFL-1), and smooth muscle cells (HISM), and all gave similar findings, suggesting that thrombin-cleavage of OPN is functionally important for a variety of cell types.

We have reported previously that both human and rat OPN are cleaved by thrombin at a single site as determined with amino acid sequence analyses (Senger *et al.*, 1989a,b). Comparisons of N-terminal amino acid sequences we obtained from the thrombin-cleaved OPNs with amino acid sequence derived from cDNA sequence (Oldberg *et al.*, 1986; Kiefer *et al.*, 1989) indicate that cleavage occurs between $arg^{153}$ and $ser^{154}$ in rat OPN and $arg^{169}$ and $ser^{170}$ in human OPN (numbers refer to residues in predicted propolypeptide sequence). For both rat and human OPN, the thrombin-sensitive arg-ser bond is just six residues carboxy-terminal to the GRGDS cell-binding sequence. Moreover, cDNA sequences of both mouse OPN (Craig *et al.*, 1989) and porcine OPN (Wrana *et al.*, 1989) predict that this same thrombin-cleavage site is also present in OPN from each of these two animal species, as well. Thus, it appears that the OPN thrombin-cleavage site in close proximity to GRGDS is conserved among mammalian species, consistent with a functional role.

Cleavage of OPN within six amino acid residues of the GRGDS domain suggests a major conformational

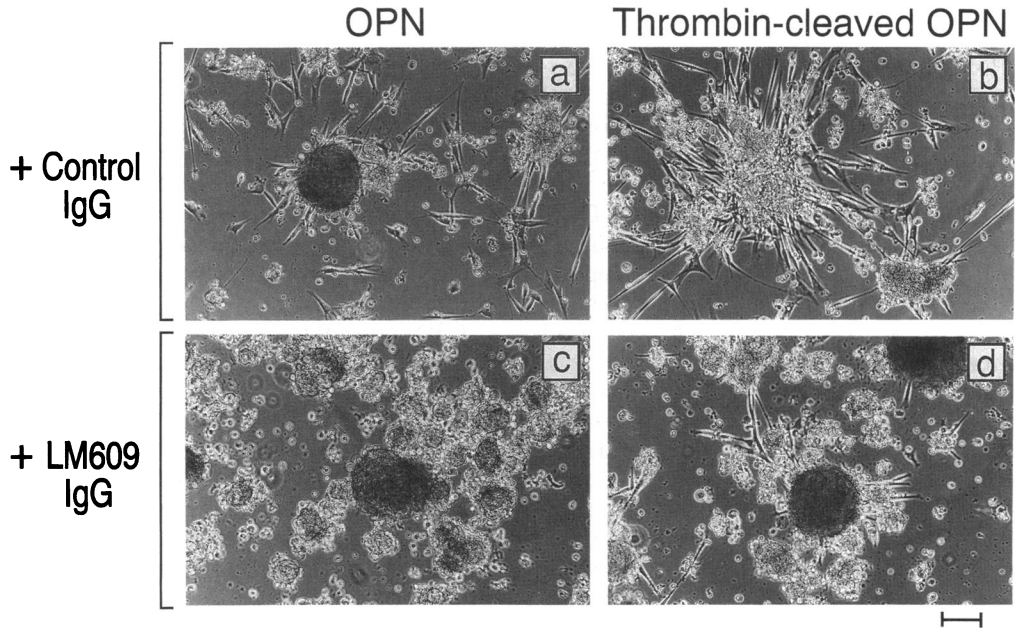


**Figure 6.** Inhibition of HFL-1 cell attachment and spreading on OPN and thrombin-cleaved OPN by $\alpha_v\beta_3$ monoclonal antibody (LM609). HFL-1 cells were plated on OPN (a and c) or thrombin-cleaved OPN (b and d), as in Figure 1, either in the presence of 10 μg/ml control mouse IgG (a and b) or 10 μg/ml purified $\alpha_v\beta_3$ monoclonal antibody LM609 (c and d). HFL-1 cell attachment and spreading on both OPN and thrombin-cleaved OPN was inhibited substantially by antibody LM609. Cells were photographed 18 h after plating. We had similar findings with T24 cells and HT1080 cells (see text). Bar, 24 μm.

change in the molecule that very likely affects the conformation of the GRGDS sequence. Moreover, and as demonstrated here, electrophoretic analyses of thrombin-cleaved OPN in nondenaturing conditions indicated that the two OPN polypeptides generated by thrombin cleavage do not remain associated. Thus, all available evidence indicates that thrombin cleavage of OPN generates two free polypeptides of Mr ~30 000, one of which has a GRGDS domain within six amino acid residues of its carboxy-terminus. We found that soluble GRGDS peptide substantially inhibited cell attachment and spreading on thrombin-cleaved OPN, as would be expected if the GRGDS domain in cleaved OPN were important for mediating cell adhesion. Moreover, antibody raised to a 20 amino acid synthetic peptide corresponding to the GRGDS domain of human OPN also substantially blocked cell attachment and spreading on both uncleaved and cleaved OPN, providing direct evidence for the importance of the GRGDS region in mediating cell attachment to uncleaved OPN and in mediating the increased cell attachment and spreading observed on thrombin-cleaved OPN. An intriguing hypothesis that could explain increased cell attachment and spreading on thrombin-cleaved OPN is that the resulting structural change in the molecule unmasks the GRGDS domain, allowing greater accessibility to cell surface receptors. Alternatively, receptors may bind both uncleaved and thrombin-cleaved OPN equally, but receptor interactions with thrombin-cleaved OPN may result in signaling that promotes greater adhesion. Additional studies will be required to distinguish among the possibilities.

Others have reported that a 28-kDa carboxy-terminal fragment of OPN, generated by digestion with endoproteinase Arg-C, promotes cell attachment and spreading (van Dijk *et al.*, 1993). N-terminal sequence analysis demonstrated that this fragment lacks the GRGDS sequence and that it is presumably very similar, although not identical, to the carboxy-terminal OPN fragment generated by thrombin-cleavage. Surprisingly, soluble GRGDSPA peptide and $\alpha_v\beta_3$ antibody reportedly blocked cell attachment to this proteolytic fragment (van Dijk *et al.*, 1993). As described above, we found with cell attachment assays performed in the presence of peptide antibody specific for the GRGDS region of OPN that essentially all of the cell binding activity of thrombin-cleaved OPN appears to be present in the GRGDS-containing, amino-terminal fragment. Purification of the carboxy-terminal fragment generated by thrombin cleavage will be required to determine if it has any adhesive properties similar to that reported for the endoproteinase Arg-C fragment.

Studies with RGD-containing peptides and RGD-containing fragments of other adhesive proteins have indicated that small fragments and peptides are generally less active than native proteins at promoting cell attachment and spreading and that small fragments and peptides bind to cells with lower affinity (Nagai *et al.*, 1991; Orlando and Cheresh, 1991; reviewed by Yamada, 1991). Such studies have provided evidence in support of the existence of important synergy sites involved in cell binding that are distinct from RGD domains but that participate cooperatively in cellular interactions with the respective RGD regions. Therefore, it is possible that the ~30-kDa GRGDS-containing fragment of OPN also has a synergy site, and because the GRGDS region is closely proximal to the carboxy-terminus, such a site must be amino-terminal to the GRGDS region of OPN. Interestingly, a 37-kDa GRGDS-containing fragment of fibronectin, which has been shown to promote cell spreading equally well as intact fibronectin, has been shown to have a synergy site amino-terminal to the GRGDS region (Nagai, 1991).

Previously, Ross *et al.* (1993) have demonstrated with specific blocking monoclonal antibody (LM609) that osteoclast binding of OPN involves interactions with the cell surface integrin $\alpha_v\beta_3$. In this study, we have demonstrated for the first time that $\alpha_v\beta_3$ binds to OPN-affinity columns in a divalent cation-dependent manner; and moreover, that this integrin similarly binds to affinity columns constructed with thrombin-cleaved OPN. Additionally, we have shown that attachment of three cell lines (HT1080, HFL-1, T24) to thrombin-cleaved OPN is in each case substantially inhibited by $\alpha_v\beta_3$-blocking monoclonal antibody (LM609), implicating $\alpha_v\beta_3$ as a major receptor both for thrombin-cleaved OPN and uncleaved OPN. Notably, the integrin $\alpha_v\beta_3$ also recognizes other RGD-containing ligands, including RGD-peptides, vitronectin, fibrinogen, von Willebrand factor, thrombospondin, fibronectin, and collagen (reviewed by Cheresh, 1991; Ruoslahti, 1991; Hynes, 1992). Thus, it is well established that the $\alpha_v\beta_3$ integrin can recognize the RGD sequence in multiple contexts; however, it is likely that these different contexts represent important functional differences. This hypothesis is supported by findings reported here that structural modifications in very close proximity to the RGD sequence of OPN markedly alters the adhesive function of this protein.

OPN present in blood plasma is cleaved by thrombin in the course of blood coagulation (Senger *et al.*, 1988), and consequently thrombin-cleaved OPN is likely present in vivo wherever the blood coagulation pathway is activated. Furthermore, the greatest quantities of thrombin-cleaved OPN would be expected in tissues where OPN expression is high and thrombin is present. Such examples very probably include most tumors. OPN is expressed at relatively high levels in a variety of human carcinomas, most often by macrophages at the tumor-stroma interface and in areas of tumor necrosis (Brown, L.F., and Senger, D.R., unpublished observation). Moreover, the presence of activated thrombin in such tumors is predicted by the observed presence of fibrin deposits (reviewed by Dvorak, 1986; Dvorak

*et al.*, 1992); thrombin-cleaved OPN is also likely to be present at sites of inflammation and tissue injury. Giachelli *et al.* (1993) have demonstrated that OPN expression increases in arterial smooth muscle cells after arterial injury, and active thrombin is likely to be generated at these sites, as well. In turn, thrombin-cleavage of OPN at sites of injury may serve to promote stronger cell adhesion and thereby contribute to regulation of the wound healing process.

Finally, multiple forms of OPN have been described, including differentially glycosylated forms (Singh *et al.*, 1990a), differentially phosphorylated species (Nemir *et al.*, 1989; Chang and Prince, 1991), and forms with and without sulphation (Nagata *et al.*, 1989). In addition, evidence has been presented for the existence of two distinct OPN mRNAs, one of which encodes a polypeptide with 14 fewer amino acid residues (Young *et al.*, 1990). The thrombin-cleavage site is predicted to be conserved in the polypeptide encoded by the alternative OPN transcript, and many of the posttranslationally modified OPN variants have been shown to be sensitive to thrombin-cleavage (Nagata *et al.*, 1989; Kubota *et al.*, 1989) as have OPNs from a variety of sources including blood plasma (Senger *et al.*, 1988), milk (Senger *et al.*, 1989b), and bone cells (Kubota *et al.*, 1989). Thus, thrombin-cleavage is likely relevant to most or all forms of OPN.

In summary, we have demonstrated that thrombin-cleavage of OPN, which occurs in close proximity to the GRGDS domain, generates two Mr ~ 30-kDa polypeptides that promote distinctly greater cell attachment and spreading than native, uncleaved OPN. As demonstrated in experiments with antibody raised to a synthetic peptide representing the GRGDS region, the GRGDS domain of thrombin-cleaved OPN, which is only six amino acid residues from the carboxy-terminus of one of the two fragments, appears to be necessary for most and possibly all of the adhesive activity. Affinity chromatography of placental integrins on supports constructed with OPN and thrombin-cleaved OPN identified $\alpha_v\beta_3$ as a receptor for both the uncleaved and thrombin-cleaved forms of OPN. Moreover, adhesion assays performed in the presence of $\alpha_v\beta_3$ blocking monoclonal antibody identified $\alpha_v\beta_3$ as a major receptor for thrombin-cleaved OPN in all three cell lines examined. The available evidence suggests that thrombin-cleavage of OPN occurs under a variety of circumstances in vivo, and data presented here predict that such cleavage is important in the regulation of OPN adhesive function.

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